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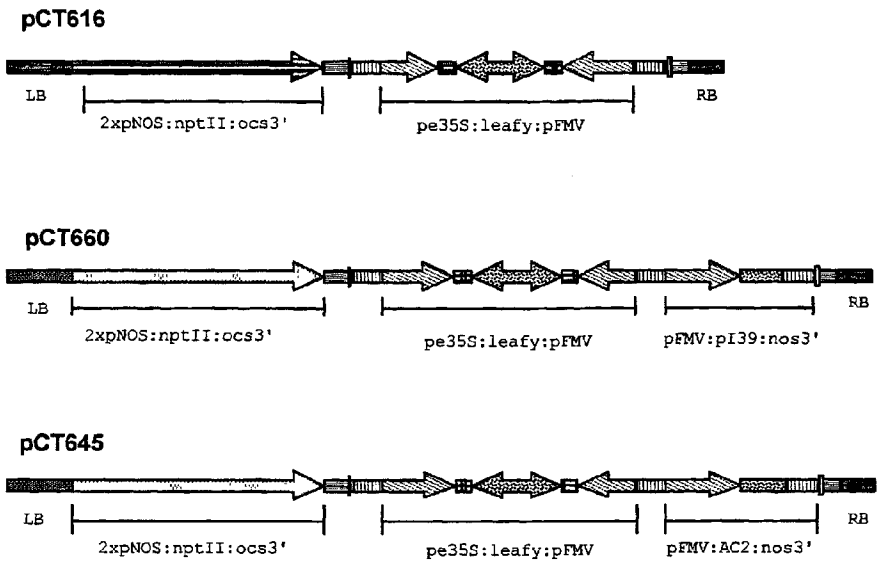
(54) Title: METHODS OF INHIBITING PLANT PARASITIC NEMATODES AND INSECT PESTS BY EXPRESSION OF NEMATODE AND INSECT SPECIFIC DOUBLE-STRANDED RNA IN PLANTS



(57) Abstract: The present invention provides methods for conferring parasitic nematode and insect pest resistance to plants, by expressing in a plant dsRNA having substantial sequence identity to an endogenous gene of the plant parasitic nematode or insect pest.

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METHODS OF INHIBITING PLANT PARASITIC NEMATODES AND INSECT PESTS BY EXPRESSION OF NEMATODE AND INSECT SPECIFIC DOUBLE-STRANDED RNA IN PLANTS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims priority to USSN 60/167,307, filed November 24, 1999, herein incorporated by reference in its entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Plant pathogens cause hundreds of millions of dollars in damage to crops in the United States annually and cause significantly more damage worldwide. Traditional plant breeding techniques have been used to develop some plants that resist specific pathogens, but these techniques are limited to genetic transfer within breeding species and can be plagued with the difficulty of introducing non-agronomic traits that are linked to pathogen resistance. Furthermore, traditional breeding has focused on resistance to specific pathogen species rather than general, or systemic, resistance to a wide spectrum of pathogens.

Plants also have certain natural cellular defense mechanisms to combat infection by pathogens, such as viruses. For example, the introduction of double-stranded RNA ("dsRNA") in eukaryotes, including plants, can result in several cellular responses, including inhibition of protein translation, activation of specific enzymes involved in dsRNA degradation, such as 2'-5' RNA synthetases and RNases, as well as systematic silencing of genes that have sequence similarity to the dsRNA (*see, e.g.*, Fire, *Trends in Genetics*, 15:358-363 (1999); Grant, *Cell* 96:303-306 (1999); Depicker & Montagu, *Curr. Opin. Cell Biol.* 9:373-382 (1997); Waterhouse *et al., Proc. Nat'l Acad. Sci USA* 95:13959-13964 (1998); Angell & Baulcombe, *EMBO J.* 16:3675-3684 (1997); *see also* Hamilton & Baulcombe, *Science* 286:950-952 (1999)). The systemic silencing of genes in plants is generally referred to as "post transcriptional gene silencing," and similar phenomena have been observed in other organisms such as nematodes, fungi, insects, and protozoa. These silencing phenomena are referred to by several terms, but may rely on

similar mechanisms and are generally sequence specific. Transcription of the target locus is not affected, while the half-life of the transcribed target RNA is significantly decreased.

Gene silencing has also been shown to occur in response to introduction of recombinant dsRNAs, typically expressed by a transgene. In this situation, gene silencing is also referred to as "dsRNA-triggered gene interference." For example, gene silencing has been induced in *Caenorhabiditis elegans*, a free living nematode (*see, e.g.*, Timmons & Fire, *Nature* 395:854 (1998); Fire *et al., Nature* 391:806-811 (1998); Hunter, *Curr. Biol.* 9:440-442 (1999); WO99/32619). *E. coli* was engineered to express a dsRNA having substantial sequence identity to a *C. elegans* target gene. When *C. elegans* ingested the bacteria, the endogenous target gene was specifically silenced. Bacteria expressing only the sense or antisense strand did not induce the same effect.

In addition to *C. elegans*, gene silencing induced by recombinant transgenes expressing dsRNA has been used in plants and other organisms to silence target endogenous genes (*see, e.g.*, Hamilton *et al., Plant J.* 15:737-746 (1998); WO99/15682)). Recombinant double-stranded RNA has also been used in plants to induce resistance to RNA viruses, using transgenes that express dsRNA having substantial sequence identity to viral genes (*see, e.g.*, Waterhouse *et al., Proc. Nat'l Acad. Sci USA* 95:13959-13964 (1998); Angell & Baulcombe, *EMBO J.* 16:3675-3684 (1997); WO98/36083; WO99/15682, and U.S. Patent No. 5,175,102). In some cases, RNA virus "amplicons" have been used to amplify the dsRNA (*see, e.g.*, Angell & Baulcombe, *EMBO J.* 16:3675-3684 (1997); WO98/36083)). In such amplicons, a cDNA of a viral genome, which includes a heterologous gene, is transcribed in the nucleus of a plant and then transported to the cytoplasm. In the cytoplasm a viral RNA dependent RNA polymerase is translated. This RNA dependent RNA polymerase then transcribes positive and/or negative transcripts from the original viral RNA, using RNA dependent RNA polymerase promoters included in the positive and/or negative transcripts.

An important goal in agriculture is to identify systems that enable plants to resist pathogens, thereby allowing for the development of systemically resistant plants through biotechnology. A need therefore exists to further develop novel technologies to control plant parasites and pathogens.

SUMMARY OF THE INVENTION

The present invention therefore provides methods of inhibiting plant pathogenic, parasitic nematodes and insect pests, by expressing in a plant or in a plant

parasitic nematode or insect pest a dsRNA having substantial sequence identity to an endogenous gene of the plant parasitic nematode or the insect pest, thereby reducing expression of the mRNA and protein encoded by the gene.

In one aspect, the present invention provides a method of conferring plant parasitic nematode or insect pest resistance to a plant cell, the method comprising the step of expressing in the plant cell a first nucleic acid comprising a first expression cassette comprising a first plant promoter operably linked to a first plant parasitic nematode or insect pest nucleic acid that produces a first transcript, which forms a double stranded RNA, either with itself or with a second complementary transcript, wherein the first nucleic acid further comprises means for enhancing RNA accumulation, thereby conferring plant parasitic nematode or insect pest resistance to the plant cell.

In another aspect, the present invention provides a method of reducing expression of a plant parasitic nematode or insect pest mRNA and its encoded protein, the method comprising the step of expressing in the plant parasitic nematode or insect pest cell a first nucleic acid comprising a first expression cassette comprising a first promoter operably linked to a first plant parasitic nematode or insect pest nucleic acid that produces a first transcript, which forms a double stranded RNA, either with itself or with a second complementary transcript, wherein the first nucleic acid further comprises means for enhancing RNA accumulation, thereby reducing expression of the plant parasitic nematode or insect pest protein.

In another aspect, the present invention provides a method of reducing expression of a plant parasitic nematode mRNA and its encoded protein, the method comprising the step of contacting the plant parasitic nematode with a double stranded RNA that is either self complementary or hybridizes with a second complementary transcript, thereby reducing expression of the plant parasitic nematode protein.

In another aspect, the present invention provides a method of reducing expression of a insect pest mRNA and its encoded protein, the method comprising the step of contacting the insect pest with a double stranded RNA that is either self complementary or hybridizes with a second complementary transcript, thereby reducing expression of the insect pest protein.

In one embodiment, the first nucleic acid is a DNA or an RNA. In one embodiment, the first transcript is self complementary.

In one embodiment, the first nucleic acid further comprises a second expression cassette. In another embodiment, the second expression cassette is the means

for enhancing RNA accumulation, comprising a second plant promoter operably linked to a viral nucleic acid encoding a protein that enhances accumulation of double-stranded RNA in the plant cell, e.g., HCPro, 2b, or AC2 protein. In another embodiment, the second expression cassette is the means for enhancing RNA accumulation, comprising a second plant promoter operably linked to a viral nucleic acid encoding an RNA dependent RNA polymerase, wherein the first transcript is operably linked to an RNA dependent RNA polymerase promoter, e.g., from tobacco mosaic virus or potato virus X. In another embodiment, the second expression cassette comprises a second plant promoter operably linked to a second plant parasitic pest nucleic acid that produces a second transcript complementary to the first transcript.

In one embodiment, the first plant pest nucleic acid encodes a gene required for nervous system function or embryonic survival. In another embodiment, the first plant pest nucleic acid encodes a vesicular acetylcholine transporter protein, a choline acetyltransferase, or a ubiquinone oxidoreductase.

In one embodiment, the plant pest is a parasitic nematode, e.g., a cyst nematode or a root-knot nematode, e.g., *Heterodera glycines*, *Globodera pallida*, *Globodera rostochiensis*, or *Meloidogyne incognita*.

In one embodiment, the plant pest is an insect pest, e.g., a piercing-sucking pest, e.g., a sap-sucking pest or a chewing pest. In another embodiment, the chewing pest is *M. sexta*.

In one embodiment, the method further comprises expressing in the plant cell a second nucleic acid comprising a second expression cassette. In another embodiment, the second expression cassette comprises a second plant promoter operably linked to a second plant pest nucleic acid that produces a second transcript complementary to the first transcript. In another embodiment, the first or the second nucleic acid is introduced into the plant cell through sexual reproduction.

In one embodiment, the plant promoter is a tissue specific promoter, e.g., a promoter active in feeding cells. In another embodiment, the plant promoter is a CaMV 35S promoter.

In one embodiment, the double stranded RNA is about 25 to 100 bp in size. In another embodiment, the double-stranded RNA has a duplex region that is at least about 50 bases in length.

In one embodiment, the plant is selected from the group consisting of pepper, tomato, squash, banana, strawberry, carrot, bean, cabbage, beet, cotton, grape, pea, pineapple, potato, soybean, yam, cucumber, melon, and alfalfa.

In one embodiment, the first transcript comprises a premature stop codon that inhibits translation of the first transcript.

In one embodiment, the first expression cassette is a plant viral vector.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Diagram of relevant regions of the vector pe35Sunc-17. Sequences complementary to *unc-17* are designated by arrows.

Figure 2. Schematic diagram of T-DNA regions from constructs that were used to generate tomato hairy root cultures. Region of vector with double arrow represents the *LFY* test sequence that is transcribed bi-directionally to generate complementary RNA's.

Figure 3. RNA blot analysis of *LFY*. Total RNA was probed with a DNA fragment derived from the leafy test sequence. The presence or absence of 23 bp RNAs is shown by a + (present) or a - (absent) beneath the transformed line number. Samples with nd below the line number were not analyzed.

Figure 4. Detection of 21-23 bp RNAs that hybridize with the *LFY* test sequence. 50 μg of total RNA was electrophoresed through a 15% acrylamide gel and electroblotted onto Duralon membrane (Stratagene, La Jolla CA). The blot was hybridized with a riboprobe derived from the *LFY* coding sequence using the conditions described by Hamilton & Baulcombe. Complete testing of all lines has been summarized in Figure 2.

Figure 5. RNA blot analysis of total RNA from *Manduca* larvae injected with water or dsRNA solutions. On the left of each panel is RNA from a mock inoculated control larvae. The other two samples in each panel are from individuals inoculated with dsRNA corresponding to the chymotrypsinogen gene (*CHY*), the S6 ribosomal protein (*S6*), and a gene coding for trypsin (*TRY*) that are described in Table 3.

DETAILED DESCRIPTION OF THE INVENTION

Introduction

The present application provides methods for inhibiting plant pests, e.g., parasitic nematodes or insects, by expressing in plants, plant parasitic nematode, or insect pest a dsRNA having substantial sequence identity to an endogenous gene of a plant parasitic nematode or insect pest. In contrast to free living nematodes such as *C. elegans*, plant parasitic nematodes are obligate parasites that cause significant damage to crops worldwide. Plant parasitic nematodes include ecto- and migratory or sedentary endo-parasites (*see, e.g.*, Appendix 2, *Plant Nematode Interactions* (Backer *et al.*, eds., 1998); *see also* Williamson & Hussey, *Plant Cell* 8:1735-1745 (1996)). Insect pests also cause significant damage to crops world-wide.

The sedentary endoparasites, family Heteroderidae, are the major source of economic loss. This family is divided into two groups: cyst nematodes (e.g., genera *Heterodera* and *Globodera*) and the root-knot nematodes (genus *Meliodogyne*). These nematodes establish permanent "feeding sites" in the plant, where root cells are morphologically transformed into large multinucleate syncytia or giant cells (*see, e.g.*, Williamson & Hussey, *Plant Cell* 8:1735-1745 (1996)). The sedentary endoparasites form elaborate, permanent associations with these cells via their specialized stylet, through which nutrients from the plant cell cytoplasm are delivered to the nematode.

In one embodiment, the present invention therefore provides dsRNA having substantial sequence identity to specific target parasite nematode genes, where the dsRNA is expressed, e.g., by transgenic plant or nematode cells. In one embodiment, the dsRNA is expressed by expression vectors comprising promoters active in plant cells (i.e., plant promoters), optionally constitutive plant promoters, preferably feeding cell specific plant promoters, or promoters active in nematode cells. The expression vectors can be either DNA or RNA vectors. In one embodiment, when the plant cells become feeding sites, dsRNA is delivered to the parasitic nematode via its stylet. The dsRNA or RNA or DNA vectors expressing the dsRNA can also be taken up directly or expressed directly by the nematode, without using the plant as an intermediate. Uptake of the dsRNA by the parasitic nematode induces a gene silencing response, which silences genes important for nematode growth, survival, or reproduction, thereby preventing nematode damage to the plant.

The invention may be used to prevent nematode damage from several genera including cyst and root-knot nematodes, as described above. Examples of cyst

nematodes include the genera *Heterodera* and *Globodera*. Species within these genera include *H. glycines*, *H. schachtii* (beet cyst nematode), *H. avenae* (cereal cyst nematode) and potato cyst nematodes *G. rostochiensis* and *G. pallida*. Root-knot nematodes include the genus *Meloidogyne*, particularly the species *M. javanica*, *M. hapla*, *M. arenaria* and *M. incognita*. Other economically important nematodes include the genus *Xiphinema*, particularly *X. index* and *X. italiae* (which transmit the grapevine fanleaf virus (GFLV) to the vine), *X. americanum* (which is of economic importance in the USA and elsewhere), *X. diversicaudatum* (which transmits arabis mosaic to raspberry and other plants, brome grass mosaic to cereals and strawberry ringspot to raspberry, rose, blackcurrant and other plants) and lesion nematodes such as the genus *Pratylenchus*, particularly *P. penetrans*, *P. bractrvurus* and *P. zeae* (which are associated with damage to maize, rice, and vegetables), *P. coffeae*, *P. bractrvurus* and others (coffee), *P. coffeae* and *P. goodeyi* (bananas), *P. brachyurus* (pineapple), and *P. thornei* (wheat). It will be noted that *Pratylenchus* sp. have wide host ranges and are not associated with any one particular crop. *Radopholus similis* is similar to members of the genus *Pratylenchus* and is also an important pest for bananas (U.S. Patent 5,494,813).

In another embodiment, the dsRNA can be targeted against piercing-sucking insects, in particular sap-sucking and chewing insects. Examples of sap-sucking insects include leafhoppers (*Homoptera*, *Cicadellidae*), aphids (*Aphididae*, *Macrosipho* sp., *Aphis* sp.), stink bugs (*Hemiptera*, *Pentatomidae*, *Euschistus* sp., *Chlorochroa ligata*, *Thyanta pallidovirens*), tarnished plant bugs (*Miridae*, *Lygus* sp.), squash bugs (*Heteroptera*, *Coreidae*, *Anasatristis*), and thrips (*Thysanoptera*, *Thripidae*, *Frankiniella* sp.) (*see, e.g.*, http://pmo.umext.maine.edu/factsht/suck.htm. Examples of chewing insects include tobacco hornworm (*Manduca sexta*).

The dsRNA is targeted to orthologs of genes described herein, e.g., genes involved in development, reproduction, motility, nervous system, sex determination, normal metabolic function and homeostasis, and the like, excluding those that perform functions specific to the family *Heteroderidae* and including genes that perform essential functions in the family *Aphididae*. Double stranded RNAs intended to confer sap-sucking insect resistance would preferentially be expressed in plant tissues on which such insects feed, e.g., primary and secondary phloem elements, and would be taken up by the insect via its sucking mechanism, e.g., its stylet (*see, e.g., Entomology and Pest Management* (3rd ed. 1999)).

In one embodiment, the dsRNA targeting a nematode or insect gene is co-expressed with a means for enhancing RNA accumulation. RNA accumulation can be enhanced in a variety of ways. For example, RNA accumulation can be enhanced via co-expressing the dsRNA and a viral protein that stabilizes RNA accumulation, e.g., HCPro from tobacco etch virus and potato virus Y, AC2 protein from African cassava mosaic virus, or orthologs of these proteins from related virus species from, e.g., cucumbo and potyviral groups, as well as other plant virus groups such as other positive strand RNA plant viruses (*see, e.g.*, Anandalakshmi *et al., Proc. Nat'l Acad. Sci. USA* 95:13079-13-84 (1998); Kasschau *et al., Cell* 95:461-470 (1998); Beclin *et al., Virology* 252:313-317 (1998); and Brigneti *et al., EMBO J.* 17:6739-6746 (1998)). These viral proteins were identified as a viral defense to gene silencing in plants. Without intending to be bound to a particular mechanism, it appears that HCPro may act by blocking the maintenance of gene silencing, whereas AC2 protein may act by preventing initiation and/or maintenance of gene silencing.

RNA accumulation can also be enhanced, e.g., via co-expression of an RNA dependent RNA polymerase and a dsRNA that comprises an RNA promoter. The RNA dependent RNA polymerase recognizes the RNA promoter and amplifies the RNA in the cytoplasm of the plant host cell. Such RNA dependent RNA polymerases thus amplify transcripts expressed by either an RNA or a DNA expression vector (*see, e.g.* Scholthof *et al., Annu. Rev. of Phytopathol.* 34:299-323 (1996), Angell & Baulcombe, *EMBO J.* 16:3675-3684 (1997) and WO98/36083). Suitable expression vectors include derivatives of plant RNA viruses in the Bromovirus, Furovirus, Hordeivirus, Potexvirus, Tobamovirus, Tobravirus, Tombusvirus, and Potyvirus groups, in particular tobacco mosaic virus, cucumber mosaic virus, tobacco etch virus, tobacco rattle virus, tomato bushy stunt virus, brome mosaic virus, potato virus X, and potato virus Y. RNA dependent RNA polymerases and RNA promoters from these viruses can be used to enhance RNA accumulation.

Suitable DNA expression vectors of the invention also include, e.g., viral-based vectors derived from plant DNA viruses, e.g., from Caulimovirus or Geminivirus, in particular, from cauliflower mosaic virus, African cassava mosaic virus, and tomato golden mosaic virus.

Suitable plant parasitic nematode and insect pest target genes include genes involved in development and reproduction, e.g., transcription factors (*see, e.g.*, Xue *et al., Science* 261:1324-1328 (1993); Finney *et al., Cell* 55:757-769 (1988)), cell cycle

regulators such as wee-1 and ncc-1 proteins (*see, e.g.* Wilson *et al., Biochim. Biophys. Acta* 1445:99-109 (1999); Boxem *et al., Developmen*t 126:2227-2239 (1999)), and embyro-lethal mutants (*see, e.g.*, Schnabel *et al., Curr. Opin. Genet. Dev.* 1:179-184 (1991)); proteins required for molting such as collagen, ChR3, and LRP-1 (*see, e.g.* Yochem *et al., Development* 126:597-606 (1999); Kostrouchova *et al., Development* 125:1617-1626 (1998); Ray *et al., Mol. Biochem. Parasitol.* 83:121-124 (1989)); genes encoding proteins involved in the motility/nervous system, e.g., acetylcholinesterase (*see, e.g.* Piotte *et al., Mol. Biochem. Parasitol.* 99:247-256 (1999); Talesa *et al., FEBS Letts.* 357:265-268 (1995); Arpagaus *et al., J. Physiol. Paris* 92:363-367 (1998)), ryanodine receptor such as unc-68 (*see, e.g.* Maryon *et al., J. Cell Sci.* 111:2885-2895 (1998); Maryon *et al., J. Cell Biol.* 134:885-893 (1996)), and glutamate-gated chloride channels or the avermectin receptor (*see, e.g.* Cully *et al., Nature* 371:707-711 (1994); Vassilatis *et al., J. Biol. Chem.* 272:33167-33174 (1997); Dent *et al., EMBO J.* 16:5867-5879 (1997)); hydrolytic enzymes required for deriving nutrition from the host, e.g., serine proteinases such as HGSP-I and HGSP-III (*see, e.g.* Lilley *et al., Mol. Biochem. Parasitol.* 89:195-207 (1997)); parasitic genes encoding proteins required for invasion and establishment of the feeding site, e.g., cellulases (*see, e.g.,* de Boer *et al., Mol. Plant Microbe Interact.* 12:663-669 (1999); Rosso *et al., Mol. Plant Microbe Interact.* 12:585-591 (1999)) and genes encoding proteins that direct production of stylar or amphidial secretions such as sec-1 protein (*see, e.g.*, Ray *et al., Mol. Biochem. Parasitol.* 68:93-101 (1994); Ding *et al., Mol. Plant Microbe Interact.* 11:952-959 (1998)); genes encoding proteins required for sex or female determination, e.g., , tra-1, tra-2, and egl-1, a suppressor of ced9 (*see, e.g.*, Hodgkin, *Genetics* 96:649-664 (1980); Hodgkin, *Genetics* 86: 275-287 (1977); Hodgkin, *Cell* 8:277-280 (1999); Gumienny *et al., Development* 126:1011-1022 (1999); Zarkower *et al., Cell* 70:237-249 (1992)); and genes encoding proteins required for maintenance of normal metabolic function and homeostasis, e.g., sterol metabolism, embryo lethal mutants (*see, e.g.*, Schnabel *et al., Curr. Opin. Genet. Dev.* 1:179-184 (1991)), and trans-spliced leader sequences (*see, e.g.*, Ferguson *et al., Genes Dev.* 10:1543-1556 (1996)). Such genes have been cloned from parasitic nematodes such as *Meliodogyne* and *Heterodera* species or can be identified by one of skill in the art using sequence information from cloned *C. elegans* orthologs (the genome of *C. elegans* has been sequenced and is available, *see* The *C. Elegans* Sequencing Consortium, *Genome Sequence of the Nematode C. elegans*, *Science* 282:2012-2018 (1998)).

Preferred target nematode genes include orthologs of the *C. elegans* genes *unc-17* (an acetylcholine transporter; *see, e.g.*, Alfonso *et al., Science* 261:617-619 (1993), *cha-1* (a choline acetyl transferase; *see, e.g.*, Alfonso *et al., J. Neurosci.* 14:2290-2300 (1994)), and *nuo-1* (NADH, a ubiquinone oxoreductase; *see, e.g.*, Tsang & Lemire, *Worm Breeders Gazette* 15.3, page 20 and 16.1, page 34; *see also* http://elegans.swmed.edu/wli/[wbg16.1p34]/ and http://elegans.swmed.edu/wli/[wbg15.3p20]/).

Suitable host plants include a broad range of plants, including, e.g., species from the genera *Allium, Anacardium, Arachis, Asparagus, Atropa, Avena, Brassica, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Cucumis, Cucurbita, Daucus, Elaeis, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Lactuca, Linum, Lolium, Lupinus, Lycopersicon, Malus, Manihot, Majorana, Medicago, Musa, Nicotiana, Olea, Oryza, Panieum, Pannesetum, Persea, Phaseolus, Pistachia, Pisum, Pyrus, Prunus, Raphanus, Ricinus, Rosa, Secale, Senecio, Sinapis, Solanum, Sorghum, Theobromus, Trigonella, Triticum, Vicia, Vitis, Vigna,* and *Zea.*

Definitions

The phrase "conferring plant pest resistance" refers to the ability of a dsRNA targeted to a specific nematode gene or insect pest gene to initiate gene silencing and prevent or reduce the effects of plant parasitic nematodes or insect pests on the plant. Such effects include stunted growth, wilting, and susceptibility to other pathogens. To examine the extent of resistance, samples or assays of nematode or insect infected plants or cells in culture expressing a particular dsRNA are compared to control samples infected with nematodes or insects but lacking expression of the specific dsRNA. Control samples (lacking specific dsRNA expression) are assigned a relative value of 100%. Pest resistance is achieved when the test value relative to the control is about 90%, preferably 50%, more preferably 25-0%. In some cases the controls express either the sense or antisense strand but not the dsRNA. The controls can also lack recombinant RNA expression altogether. Suitable assays include those described below in the Example section, e.g., infection of either transgenic plants or hairy root cultures with J2 juveniles of *M. incognita*, and then observing the formation of galls and production of egg masses, or by measuring plant growth and yield, or *in situ* RNA hybridization in feeding nematodes using digoxygenin-labeled RNA probes corresponding to the target RNA

(Seydoux & Fire in: *Modern Biological Analysis of an Organism* (Epstein & Shakes, eds., 1995)).

The term "plant" includes whole plants, shoot vegetative organs/structures (e.g. leaves, stems and tubers), roots, flowers and floral organs/structures (e.g. bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (e.g. vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous.

A premature stop codon "inhibits" translation of the transcript, resulting in a lack of functional protein.

"Plant pest" refers to parasitic nematodes and insects pests such as piercing-sucking insects, e.g. sap-sucking and chewing insects.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill

will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M)

(*see, e.g.*, Creighton, *Proteins* (1984)).

The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a

polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions and in most plant tissues. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

A "plant promoter" is a promoter capable of initiating transcription in plant cells.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, *e.g.*, a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (*e.g.*, a fusion protein).

An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter. The expression vector can be an RNA or a DNA vector.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., at least about 60%, preferably 65%, 70%, 75%, preferably 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Such sequences that have at least about 60% identity are then said to be

"substantially identical." This definition also refers to the compliment of a test sequence. Preferably, the identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

In the case of both expression of transgenes and inhibition of endogenous genes (e.g., by antisense, sense, or dsRNA expression) one of skill will recognize that the inserted polynucleotide sequence need not be identical, but may be only "substantially identical" to a sequence of the gene from which it was derived.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g.,* *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used,

with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

Double stranded RNA

The dsRNA of the invention, which has substantial identity to a target nematode gene, can be expressed separately as two transcripts, one encoding the sense strand and one encoding the antisense strand. Genes encoding these transcripts can be introduced into the plant on a single vector or different vectors. In one embodiment, one homozygous parent plant expresses the antisense transcript and the second homozygous parent plant expresses the sense transcript. When the plants are crossed, the resulting hybrid expresses both transcripts. In another embodiment, the dsRNA is expressed as a single self-complementary RNA that forms an internal hairpin. RNA duplex formation may be initiated either inside or outside the cell. The dsRNA may be partially or fully double-stranded. The RNA can be enzymatically or chemically synthesized, either *in vitro* or *in vivo*.

The dsRNA of the invention may optionally comprise a stop codon, for prevention of translation of the transcript.

The dsRNA sequences disclosed here can be used to inhibit target gene expression in plant pests, e.g., parasitic nematodes or insect pests. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter or promoters such that a sense and an antisense strand of RNA will be transcribed. The construct is then transformed into plants or pests and the dsRNA is produced. The nucleic acid segment to be introduced generally will be substantially identical (i.e., have at least about a minimum percent identity) to at least a portion of the endogenous target gene or genes to be inhibited. This minimal identity will typically be greater than about 60%, but a higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 80% is preferred, though about 95% to absolute identity would be most preferred. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other genes within a family of genes exhibiting identity or substantial identity to the target gene.

The introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments may be equally effective. Normally, a double stranded region of the dsRNA has a length of at least about 25 nucleotides, optionally a sequence of about 25 to about

50 nucleotides, optionally a sequence of about 50 to about 100 nucleotides, optionally a sequence of about 100 to about 200 nucleotides, optionally a sequence of about 200 to about 500, and optionally a sequence of about 500 to about 1000 or more nucleotides, up to molecule that is double stranded for its full length, corresponding in size to a full length endogenous plant parasite transcript.

The dsRNA may comprise single stranded regions as well, e.g., the dsRNA may be partially or fully double stranded. The total length of the dsRNA (either single or double stranded) is at least about 25 nucleotides, optionally a sequence of about 25 to about 50 nucleotides, optionally a sequence of about 50 to about 100 nucleotides, optionally a sequence of about 100 to about 200 nucleotides, optionally a sequence of about 200 to about 500, and optionally a sequence of about 500 to about 1000 or more nucleotides, up to a length that corresponds in size to a full length endogenous plant parasite transcript.

The dsRNA of the invention is preferably present or expressed in feeding cells, and therefore is taken up by the parasitic nematode or insect pest via its stylet. In one embodiment, for efficient uptake of the dsRNA via the stylet, the dsRNA is no more than about 25-100 bp

A number of gene regions can be targeted to suppress target gene expression. The targets can include, for instance, the coding regions, introns, sequences from exon/intron junctions, 5' or 3' untranslated regions, and the like.

For specific target genes of the invention, antisense and sense silencing can also be used to inhibit parasitic nematodes. Normally, the size ranges and other parameters noted above for dsRNA regulation is used for antisense and sense silencing.

Cloning of target nucleic acids

Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, (1989) or *Current Protocols in Molecular Biology* Volumes 1-3 (Ausubel, *et al.*, eds. 1994-1998).

The isolation of nucleic acids corresponding to target parasitic nematode genes may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatamers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired organ, such as flowers, and a cDNA library which contains the target gene transcript is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which target genes or homologs are expressed.

The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned target gene disclosed here, such as a *C. elegans* ortholog. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. Alternatively, antibodies raised against an target polypeptide can be used to screen an mRNA expression library.

Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of the target genes directly from genomic DNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. For a general overview of PCR see *PCR Protocols: A Guide to Methods and Applications*. (Innis *et al.*, eds. 1990). Appropriate primers and probes for identifying target sequences from plant parasitic nematodes are generated from comparisons of the sequences provided here (e.g. *C. elegans* orthologs).

Polynucleotides may also be synthesized by well-known techniques as described in the technical literature (*see, e.g.*, Carruthers *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams *et al.*, *J. Am. Chem. Soc.* 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Promoters and expression vectors

As described below, an expression vector expressing the dsRNA of the invention can be introduced into a plant by any suitable method. For example, the dsRNA can be introduced into a plant via stable transformation with *Agrobacterium*, particle bombardment, electroporation, or transduction with a viral particle. A suitable expression vector is therefore selected according to the desired method of plant transformation.

In one embodiment, the dsRNA is expressed via a DNA expression vector. Such expression vectors comprise DNA dependent RNA polymerase promoters that are active in plant cells, e.g., constitutive plant promoters such as those described below (e.g., the nopaline synthase promoter, Sanders *et al., Nuc. Acids Res.* 15:1543-1558 (1987); or the CaMV 35S promoter, Urwin *et al., Mol. Plant Microbe Interact.* 10:394-400 (1997)) or tissue specific plant promoters such as those described below. Preferably the plant promoters are expressed in parasitic nematode feeding sites, e.g., the CaMV 35S promoter (Urwin *et al., Mol. Plant Microbe Interact.* 10:394-400 (1997); WO92/21757; WO93/10251; WO93/18170; WO94/10320; WO94/17194, US Patent No. 5,750,386, US Patent No. 5,612,471; Yamamoto *et al., Plant Cell* 3:371-382 (1991)). Other suitable promoters include those that are expressed in plant pests, e.g., insects and nematodes. Alternatively, the dsRNA can be expressed and then directly administered to the plant, insect, or nematode.

In another embodiment, the expression vector (either DNA or RNA) co-expresses a protein such as HCPro, 2b, or AC2 protein, which stabilize RNA transcripts, as described above.

In another embodiment, the dsRNA is expressed via an RNA expression vector. The RNA expression vector encodes an RNA dependent RNA polymerase active in plant cells, and the dsRNA is transcribed via an RNA dependent RNA polymerase promoter active in plant cells. Suitable RNA dependent RNA polymerases and their corresponding promoters and expression vectors are derived, e.g., from potato virus X (Chapman *et al., Plant J.* 2:549-557 (1992), tobacco mosaic virus (*see, e.g.*, Dawson *et al., Virology* 172:285-292 (1989)), tobacco etch virus (*see, e.g.*, Dolja *et al., Proc. Nat'l Acad. Sci. USA* 89:10208-10212 (1992)), tobacco rattle virus (*see, e.g.*, Ziegler-Graff *et al., Virology* 182:145-155 (1991)), tomato bushy stunt virus (*see, e.g.*, Scholthof *et al., Mol. Plant Microbe Interact.* 6:309-322 (1993)), brome mosaic virus (*see, e.g.*, Mori *et al., J. Gen. Virol.* 74:1255-1260 (1993)),. Such expression vectors are prepared using

techniques known to those of skill in the art, e.g., by using bacterial RNA polymerases such as SP6 and T7 followed by manual inoculation, or by introduction of the vectors into plants by *Agrobacterium*-mediated transformation (Angell & Baulcombe, *EMBO J.* 16: 3675-3684 (1997)).

In another embodiment, optionally, a DNA expression vector also comprises a gene encoding an RNA dependent RNA polymerase active in plant cells and the dsRNA transcript comprises an RNA dependent RNA polymerase promoter, as described above. The RNA dependent RNA polymerase is then used to amplify the dsRNA (either the positive and/or the negative strand).

In another embodiment, the dsRNA is expressed via a DNA expression vector derived from a plant DNA virus, e.g., cauliflower mosaic virus (*see, e.g.*, Futterer & Hohn, *EMBO J.* 10:3887-3896 (1991), African cassava mosaic virus (*see, e.g.*, Ward *et al., EMBO J.* 7:1583-1587 (1988)) and the tomato golden mosaic virus.

In the present invention, a plant, insect, or nematode promoter may be employed which will direct expression of the gene, e.g., in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive plant promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumafaciens*, and other transcription initiation regions from various plant genes known to those of skill. Such genes include for example, *ACT11* from *Arabidopsis* (Huang *et al. Plant Mol. Biol.* 33:125-139 (1996)), *Cat3* from *Arabidopsis* (GenBank No. U43147, Zhong *et al.*, *Mol. Gen. Genet.* 251:196-203 (1996)), the gene encoding stearoyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe *et al. Plant Physiol.* 104:1167-1176 (1994)), *GPc1* from maize (GenBank No. X15596, Martinez *et al. J. Mol. Biol.* 208:551-565 (1989)), and *Gpc2* from maize (GenBank No. U45855, Manjunath *et al.*, *Plant Mol. Biol.* 33:97-112 (1997)).

Alternatively, the promoter, e.g., a plant promoter, may direct expression of the dsRNA in a specific tissue, organ or cell type (i.e. tissue-specific promoters) or may be otherwise under more precise environmental or developmental control (i.e. inducible promoters). Examples of environmental conditions that may effect transcription by inducible promoters include pathogen challenge, anaerobic conditions, elevated temperature, the presence of light, or spraying with chemicals/hormones. One of skill will recognize that a tissue-specific promoter may drive expression of operably

linked sequences in tissues other than the target tissue. Thus, as used herein a tissue-specific promoter is one that drives expression preferentially in the target tissue or cell type, but may also lead to some expression in other tissues as well.

A number of tissue-specific promoters can also be used in the invention. For instance, plant promoters that direct expression of nucleic acids in roots and feeding cells can be used. In particular, such promoters are useful for using the methods of the invention to inhibit nematode endoparasites that live in roots. The root-specific *ANR1* promoter is suitable for use in the present invention (Zhang & Forde, *Science* 279:407 (1998)). The wound specific promoter *wun-1* from potato can be used, as it respond to intracellular root migration by *Globodera* sp. (*see, e.g.*, Hansen *et al., Physiol. Mol. Plant Pathol.* 48:161-170 (1996)). Other genes that demonstrate parasitic nematode feeding-cell specific expression have been reported, and their promoters are suitable for use in the present invention (*see, e.g.*, Bird *et al., Mol. Plant Microbe Interact.* 7:419-424 (1994); Gurr *et al., Mol. Gen. Genet.* 226:361-366 (1991)); Lambert *et al., Nucl. Acids. Res.* 21:775-776 (1993); Opperman *et al., Science* 263:221-223 (1994); Van der Eycken *et al., Plant J.* 9:45-54 (1996); and Wilson *et al., Phytopathology* 84:299-303 (1992)). Phloem specific promoters, which can be used to express the dsRNA of the invention for uptake by sap-sucking insects, include those referenced in Shi *et al., J. Exp. Bot.* 45:623-631 (1994).

If proper polypeptide expression is desired, for example, with the co-expressed RNA dependent RNA polymerases or viral proteins that enhance RNA stability, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA. The vectors of the invention can also comprise a transcriptional terminator.

The vector comprising the sequences (e.g., promoters or dsRNA coding regions) from genes of the invention will typically comprise a marker gene that confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

Plant transformation

Expression vectors of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the expression vector may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the expression vectors can be introduced directly to plant tissue using ballistic methods, such as particle bombardment. In addition, the constructs of the invention may be introduced in plant cells as DNA or RNA expression vectors or viral particles that co-express an RNA dependent RNA polymerase.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of expression vectors using polyethylene glycol precipitation is described in Paszkowski *et al.*, *EMBO J.* 3:2717-2722 (1984). Electroporation techniques are described in Fromm *et al.*, *Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein *et al.*, *Nature* 327:70-73 (1987).

Alternatively, the expression vectors may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature (*see, e.g.*, Horsch *et al.*, *Science* 233:496-498 (1984); Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* 80:4803 (1983) and *Gene Transfer to Plants* (Potrykus, ed. 1995)).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype such as enhanced resistance to pathogens. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans *et al.*, *Protoplasts Isolation and Culture*, *Handbook of Plant Cell Culture*, pp. 124-176 (1983); and Binding, *Regeneration of Plants*, *Plant Protoplasts*, pp. 21-73 (1985). Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration

techniques are described generally in Klee *et al.*, *Ann. Rev. of Plant Phys.* 38:467-486 (1987).

The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Thus, the invention has use over a broad range of plants, including pepper, tomato, squash, banana, strawberry, carrot, bean, cabbage, beet, cotton, grape, pea, pineapple, potato, soybean, yam, cucumber, melon, and alfalfa, as well as other species described herein.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants, if such a technique is used, and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Using known procedures one of skill can screen for plants of the invention by detecting the effect of the dsRNA of the invention in target nematodes and insects, either using *in vitro* assays such as a hairy root culture, or *in vivo* assays such as transgenic plants. Means for directly and indirectly detecting and quantitating dsRNAs *in vitro* and in cells are well known in the art.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example 1

Example 1 describes isolation of the *unc-17* gene of *M. incognita* and the production of *unc-17* specific dsRNA within plant cells, tissues, and whole plants. Several means of assaying plant tissues for production of dsRNA and the effects of exposure to dsRNA on parasitic populations on *M. incognita* are included.

Isolation of unc-17 from M. incognita

The *unc-17* gene of *C. elegans* encodes a putative vesicular acetylcholine transporter (Alfonso *et al., Science* 261:617-619 (1993)). If this gene is absent or mutated in *C. elegans*, proper development is arrested leading to loss of viability at the embryo level. Loss of function of this gene in other free living or parasitic nematodes as well as other organisms such as sap-sucking insects would also be likely to lead to reduced fitness or death. *Unc-17*, therefore represents an appropriate target for dsRNA interference that falls into a class of genes considered as being required for proper function of the nervous system. The complete *C. elegans* unc-17 polypeptide sequence is available from the Swissprot database under accession number P34711. The integral membrane protein encoded by this gene contains conserved regions that are present in diverse organisms. A predicted protein alignment of the unc-17 protein as well as those from close relatives was produced by the method of Thompson *et al., Nucl. Acids Res.* 22:4673-4680 (1994)), as follows. Closest relatives present in protein databases were obtained by blastp (Altshul *et al., Nuc. Acids Res.* 25:3389-3402 (1997)) of the *C. elegans* unc-17 protein. Alignment of unc-17 polypeptide and close relatives was performed using the Clustal W multiple sequence alignment software (Thompson, *supra*). Sequences are derived from mouse (gi|2598922|Mus), rat (gi|559766|Rattus), human (gi|2136357|human), electric ray (gi|5738197|Torpedo), and fruit fly (gi|2625056|Drosophila). Based on the alignment, several conserved regions are revealed that can be utilized for cloning other *unc-17* gene-family members by sequence homology.

The conserved regions present in the multiple sequence alignment were used to produce four degenerate oligonucleotides that were chosen using the CODEHOP sequence prediction algorithm (Rose *et al., Nucl. Acids Res.* 26:1628-1635 (1998)) and are shown in Table 1. In this method, the unc-17 protein was weighted by a factor of two relative to the other sequences in the alignment shown, and the oligonucleotides were

biased towards the codon preferences of nematodes. Some nucleotides were altered to change codons to the corresponding *C. elegans* amino acid at that position.

To clone the *M. incognita* ortholog of *unc-17*, these four degenerate primers are synthesized (sequences 1-4, Table 1) and the polymerase chain reaction (PCR) is used with these primers to amplify the corresponding *unc-17* gene from *Meloidogyne incognita* or other nematode. Either cDNA or chromosomal DNA prepared from J2 juveniles is used as a template. Thermal cycling conditions corresponding to those described in Rose *et al., supra* are used. Each primer pair should yield a PCR product of between 385 and 410 base pairs.

The resulting PCR product of the correct size is cloned directly into pCRII-TOPO (Invitrogen, Carlesbad CA) according to manufacturers instructions. At least four clones are selected for sequence analysis to identify a single clone that has sequence similarity to *unc-17* with no mutations artificially introduced by the Taq polymerase and that is oriented so the *XbaI* recognition sequence derived from the pCRII-TOPO vector is adjacent to the 5' end of the coding strand.

Production of dsRNA and assaying gene function in vitro

The pCRII-TOPO vector contains SP6 and T7 promoter sequences that can be used for the synthesis of RNA *in vitro* using purified SP6 and T7 polymerases (Promega Corp). RNA transcripts from *unc-17* cloned from *M. incognita* and *C. elegans* using primer sequences 1-4 into pCRII-TOPO are digested with either *KpnI* or *XbaI*. The product digested with *XbaI* is incubated with SP6 RNA polymerase and ribonucleotides to synthesize the (-) sense strand RNA, and the product digested with *KpnI* is incubated with T7 polymerase and ribonucleotides to synthesize the (+) sense strand RNA. After DNA template removal with RNase free DNase I, the two RNAs are denatured and annealed to one another. These RNAs are used for microinjection of *C. elegans* larvae and adult hermaphrodites and *M. incognita* J2 larvae and feeding larvae and adults to test for silencing of the endogenous *unc-17* locus. Approximately $1x10^6$ copies of the *in vitro* transcribed and annealed dsRNA are used for each inoculation as estimated based on ethidium bromide staining of the dsRNA solution and degree of liquid displacement in inoculated individuals. Inoculations are performed by the method of Fire *et al. Nature* 391:806-811 (1998) with modifications for inoculation of *M. incognita* individuals feeding on roots.

In the case of *M. incognita*, suppression of unc-17 protein activity in preparasitic larvae and parasitic larvae and adults is determined in three ways: (1) by measuring chemotactic behavior of injected animals toward root exudates; (2) by the frequency with which injected individuals successfully establish feeding sites on tomato roots is determined; and (3) by determining the number of viable progeny from injected animals when feeding on non-transgenic tomato roots. Chemotactic behavior is measured by placing injected embryos, or J2 larvae, 1 cm from a tomato root tip placed on agar media and measuring the time taken to reach the root tip. Similarly, the frequency with which individuals successfully establish feeding sites in measured by placing a known number of inoculated individuals on the root surface and counting the numbers of roots that display enlargement of cells in the vascular bundle and tip swelling. Fecundity is measured by inoculation of female parasitic second through fourth stage juveniles residing on tomato roots with dsRNA followed, after a constant time interval, by isolation of eggs by the method of Hansen *et al.*, *Physiol. Mol. Plant Path.* 48:161-170 (1996) from injected individuals. The number of eggs produced in control verses individuals injected with *unc-17* specific double stranded RNA is determined and their viability is assayed qualitatively by hatching in water. In most cases where plant tissue is required it is preferable to use tomato root cultures produced by *Agrobacterium rhizogenes* by the method of Hansen *et al., Physiol. Mol. Plant Path* 48:161-170 (1996).

In the case of *C. elegans*, unc-17 protein activity is measured indirectly as percentage of viable embryos produced from juveniles injected with control treatments and *unc-17* dsRNA.

Generation of plant expression vectors for plant transformation

The sequence of the first PCR product is used to generate two new specific oligonucleotide primers that amplify a product from the 5' end of the coding strand of between 150 and 200 bp in length. An *XhoI* site is incorporated into the new primer that is at the 5' end of the coding strand for later cloning steps. This second PCR product is also cloned into pCRII-TOPO. At least four PCR clones are sequenced from the second product to verify that no mutations are present in at least on sequence and that the 5' end of the coding strand is oriented adjacent to the unique *XbaI* site of the pCRII-TOPO vector.

The correct clone of PCR reaction 1 is digested with *SpeI* and *XbaI*, and the correct clone from PCR reaction 2 is digested with *SpeI* and *XhoI*. DNA fragments

containing the small PCR inserts are gel purified and ligated in a single reaction into the larger fragment of pCT583 that has been digested with *XhoI* and *XbaI*. This cloning functionally links the enhanced 35S promoter of cauliflower mosaic virus and the petunia rubisco small subunit E9 polyadenylation signal to both PCR products in a three part ligation, thus creating pe35Sunc-17, a palindromic *unc-17* sequence linked to elements that direct expression in plant cells (Figure 1). It is not necessary in this technique to produce a functional protein because the suppression mechanism is via RNA.

The intermediate vector pe35Sunc-17 is then digested with *BamHI* and *BglII* to liberate the promoter, palindromic, and terminator sequences. The purified fragment containing these sequences is then cloned into the unique *BamHI* site of the plant-transformation vector pWTT2144. The recombinants are selected by blue white screening for the *lacZ* gene product followed by restriction enzyme analysis to verify recombinant clones.

The resulting binary vector plasmid (pBINunc-17) is transferred to *Agrobacterium tumefaciens* strain LBA4404 by electroporation of the plasmid DNA directly, and stable transformants of *Lycopersicon esculentum* cv. Bonnie Best are regenerated by standard cotyledon transformation methods using LBA4404 (*see e.g.* McCormick *et al.*, *Plant Cell Reports* 5:81-84 (1986)). Alternatively, the plasmid pBINunc-17 is electroporated into *Agrobacterium rhizogenes* (ATCC #15834) for the production of transgenic hairy root cultures from *L. esculentum* cv. Bonnie Best. by the method of Urwin *et al.*, *Plant J.* 8,121-131 (1995). Similar plant transformation vectors are made and introduced into *A. tumefaciens* and *A. rhizogenes* from the PCR product alone in either orientation for use as control vectors and for the production of dsRNA through sexual crosses of transformed lines homozygous for each respective sense or antisense transcript.

Production of dsRNA in transgenic plants and analysis of transgenic plant resistance to M. incognita

Prior to infection assays on transformed individuals, the presence and stability of dsRNA is assayed in both transgenic plants and transformed root cultures by: (1) RNA blot analysis; (2) binding to CF-11 cellulose; (3) resistance to RNase A in high salt, and (4) sensitivity to *E. coli* RNase III (Nicholson, *Prog. in Nucl. Acid Res. and Mol. Biol.* 52:1-51 (1996)). Similar non-palindromic transgenic plants generated by the introduction of PCR product 1 in either orientation are analyzed in parallel as controls.

Transformed lines expressing the highest quantities of dsRNA are then chosen for further analysis.

Infection assays are performed by inoculation of whole plants and hairy roots with J2 juveniles of *M. incognita*. Transgenic pBINunc-17 plants and hairy root cultures established with pBINunc-17 are used as well as control transgenic plants expressing the (+) or (-) strand alone. Infections are performed on plants growing in potting media or under monoxenic conditions. These J2 larvae are isolated from hydroponically grown tomato plants infected with *M. incognita* (Lambert *et al., Phytopathology* 82:512-515 (1992)), or alternatively they may be obtained from surface sterilized egg masses that are isolated from female nematodes feeding on infected plants. Juveniles are placed in the soil or in proximity to actively growing root tips and are allowed to establish infections by penetration of the root tip and establishment of a feeding site.

Specific whole plant resistance to *M. incognita* is determined by measuring the rate of plant growth and yield, the formation of root knots or galls, and by analysis of nematode population growth rates as expressed in egg masses per plant and the number of individuals per gram of soil. Analysis of individual nematode growth rates as determined by mean body volume per nematode over time can is estimated by direct measurement of acid fuschin-stained individuals with computer-assisted morphometric analysis (Atkinson *et al., J. Nematol.* 28:209-215 (1996)).

Meloidogyne incognita unc-17 protein function is assayed by several methods to establish that the measured reduction of gall formation and increased yield is due to loss of functional unc-17 protein. These assays are performed on both feeding females and their progeny to determine if the predicted nematode phenotype resulting from loss of unc-17 protein is manifested to any extent in either the primary or subsequent generations. Silencing of *unc-17* RNA is verified in feeding nematodes and their progeny by *in situ* RNA hybridization using digoxygenin-labeled RNA probes corresponding to *unc-17* to localize *unc-17* mRNA (Seydoux & Fire in: *Modern Biological Analysis of an Organism* (1995)). Indirect immuno-fluorescence microscopy with anti-unc-17 antisera is also performed on fixed and sectioned nematodes of several stages after infection by the method of Alfonso *et al., Science* 261:617-619 (1993). Indirect evidence for loss of unc-17 protein function is also obtained by comparison of nematode phenotypes to the phenotype produced by pharmacological inhibitors of

acetylcholine-mediated neurotransmission such as levamisol (Lewis *et al., Mol. Pharmacol* 31:185-193 (1987)).

Table 1: Sequences of degenerate polynucleotides used to amplify *unc-17* from *Meloidogyne incognita*. Yields a predicted product of 397 bp.

```
gi|2136357|human    G  A  L  T  T  C  N  I  P  L  A  F  L  E  P  T  I  A
gi|5738197|Torpedo  G  A  L  T  T  C  N  I  P  L  A  F  L  E  P  T  I  S
gi|2625056|DrosophilG  A  M  T  M  S  N  V  A  L  A  F  L  E  P  T  I  S
gi|1078901unc-17    G  A  L  I  M  A  N  V  S  L  A  F  L  E  P  T  I  T
sequence 1                              5'-GTTCCACTGGCTTTTCTGgarccnacnat-3'
sequence 2                  5'-CTATGGCAAATGTTCCACTGGCTttyytngarcc-3'

gi|2598922|Mus   V  Y  A  I  A  D  I  S  Y  S  V  A  Y  A  L  G  P  I  V
gi|559766|Rattus V  Y  A  I  A  D  I  S  Y  S  V  A  Y  A  L  G  P  I  V
gi|2136357|human V  Y  A  I  A  D  I  S  Y  S  V  A  Y  A  L  G  P  I  V
gi|5738197|TorpedV  Y  A  I  A  D  I  S  Y  S  V  A  Y  A  L  G  P  I  M
gi|2625056|DrosopI  Y  A  I  A  D  I  S  Y  S  I  A  Y  A  V  G  P  I  I
gi|1078901unc-17 V  Y  A  I  A  D  I  S  Y  S  L  A  Y  A  F  G  P  I  I
sequence 3      3'-atrcgntadcgACTATAAAGAATAAGAGATCGAATACG-5'
sequence 4            3'-tadcgnctrtaAAGAATAAGAGATCGAATACGAAAAC-5'
```

Example 2

Example 2 describes the isolation of an ortholog in *M. incognita* of the *nuo-1* gene of *C. elegans*, and its use in dsRNA interference in transgenic plants.

The *nuo-1* gene encodes the active site subunit of respiratory complex I (NADH ubiquinone oxidoreductase) that participates in the oxidation of NADH and transfer of electrons to ubiquinone (Tsang & Lemire, *Worm Breeders Gazette* 15.3, p. 20; 16.1, p. 34). The predicted protein sequence is available from GenBank accession #Z50109. As described in Example 1, a multiple sequence alignment was produced for *nuo-1* using several closely related genes (Table 2). Closest relatives present in protein databases obtained by blastp of the *C. elegans* nuo-1 protein. Alignment of nuo-1 polypeptide and close relatives is performed using the Clustal W multiple sequence alignment software. Highly conserved regions are shown in bold. Sequences are derived from human (gi|5138912|Human), cow (gi|108828|bovine), *C. elegans* (gi|3874218|C. elegans nuo-1), *Neurospora* (gi|83779| Neurospora), *Aspergillus* (gi|1556376|Aspergillus niger), slime mold (gi|1408306|Dictyostelium), Potato (gi|639834|Potato), and *Paracoccus* (gi|150598|P. denitrificans). Degenerate polynucleotides corresponding to sequences 5-8 in Table 2 were used to clone the sequences from either chromosomal

DNA or cDNA with the polymerase chain reaction. The cloning steps are performed essentially as in Example 1 to generate plant transformation vectors.

Accumulation of dsRNA is measured in plants by the methods detailed in Example 1 and plant resistance is measured with infection assays performed with J2 juveniles as in Example 1.

The activity of nuo-1 protein in feeding nematodes and their progeny is assayed by measuring respiration rates with an oxygen electrode and isolated nematodes. Respiration is also measured *in situ* by microscopy with G6 rhodamine, a fluorescent dye that accumulates in mitochondria generating an active membrane potential. Presence of dye in and around mitochondria may therefore be used as an assay for proper mitochondrial function (Felkai *et al., EMBO J* 18:1783-1792 (1999); Johnson *et al., J. Cell. Biol.* 88:526-535 (1981)). *In situ* RNA hybridizations with feeding *M. incognita* and their progeny are also performed as described in Example 1, with digoxygenin labeled *nuo-1* RNA.

Table 2: Sequences of degenerate polynucleotides used to amplify *nuo-1* from *Meloidogyne incognita.* PCR using these primers should yield products of 302 or 357 bp.

```
gi|5138912|Human          V  V  N  A  D  E  G  E  P  G  T  C  K  D  R  E  I
gi|108828|bovine          V  V  N  A  D  E  G  E  P  G  T  C  K  D  R  E  I
gi|3874218|C. elegans NU  V  V  N  A  D  E  G  E  P  G  T  C  K  D  R  E  I
gi|83779| Neurospora      V  V  N  A  D  E  G  E  P  G  T  C  K  D  R  E  I
gi|1556376|Aspergillus n  V  V  N  A  D  E  G  E  P  G  T  C  K  D  R  E  I
gi|1408306|Dictyostelium  V  I  N  A  D  E  G  E  P  G  T  C  K  D  R  E  I
gi|639834|Potato          V  V  N  A  D  E  S  E  P  G  T  C  K  D  R  E  I
gi|150598|P. denitrifica  V  I  N  A  D  E  S  E  P  A  T  C  K  D  R  E  I
sequence 5                              5'-TGAAGGAGAACCAGGAACAtgyaargaymg-3'
sequence 6                           5'-CTGATGAAGGAGAACCAGGAacntgyaarga -3'

gi|5138912|Human          A  Y  I  C  G  E  E  T  A  L  I  E  S  I  E  G  K  Q
gi|108828|bovine          A  Y  I  C  G  E  E  T  A  L  I  E  S  I  E  G  K  Q
gi|3874218|C. elegans NUA  Y  I  C  G  E  E  T  A  L  I  E  S  L  E  G  K  Q
gi|83779| Neurospora      A  Y  V  C  G  E  E  T  S  L  I  E  S  L  E  G  K  P
gi|1556376|Aspergillus nA  Y  V  C  G  E  E  T  S  L  I  E  S  I  E  G  K  A
gi|1408306|DictyosteliumA  Y  I  C  G  E  E  T  A  L  I  G  S  I  E  G  K  Q
gi|639834|Potato          A  Y  I  C  G  E  E  T  A  L  L  E  S  L  E  G  K  Q
gi|150598|P. denitrificaA  Y  I  C  G  E  E  T  A  L  L  E  S  L  E  G  K  K
sequence 7                           3'-ccnctyctytgTCGAGACTAACTTAGATAACTTCC-5'
 (5'-CCTTCAATAGATTCAATCAGAGCTgtytcytcncc-3')
```

```
gi|5138912|Human            P  P  F  P  A  D  V  G  V  F  G  C  P
gi|108828|bovine            P  P  F  P  A  D  V  G  V  F  G  C  P
gi|3874218|C. elegans NUO-  P  P  F  P  A  D  I  G  L  F  G  C  P
gi|83779| Neurospora        P  P  F  P  A  A  V  G  L  F  G  C  P
gi|1556376|Aspergillus nig  P  P  F  P  A  A  V  G  L  F  G  C  P
gi|1408306|Dictyostelium    P  P  F  P  A  M  A  G  L  Y  G  C  P
gi|639834|Potato            P  P  F  P  A  N  A  G  L  Y  G  C  P
gi|150598|P. denitrificans  P  P  F  P  A  G  A  G  L  Y  G  C  P
sequence 8              3'-ggnggnaarggTCGACTATATCCTGACAAA -5'
5'-AAACAGTCCTATATCAGCTggraanggngg-3'
```

Example 3

This example describes the use of plant-viral inhibitors of gene silencing to allow accumulation of sufficient quantities of dsRNA in plant cells to surpass the threshold necessary for triggering of RNA silencing in plant parasitic nematodes.

Some viral proteins have been identified that have the ability to suppress post-transcriptional gene silencing in virus-infected plants or in plants that are transformed with them (*see, e.g.*, Kasschau *et al., Cell* 95:461-470 (1998); Brigneti *et al., EMBO J.* 17:6739-6746 (1998)). The mechanism through which this occurs is not well understood, but it is likely that they exert their influence through inhibition of a natural defense mechanism against viruses. These factors can be used as a means of promoting high expression of dsRNA in plant cells by co-expression of the viral suppressor of gene silencing with self-complementary RNA transcripts that would otherwise trigger gene silencing. Two examples of such proteins from the potyvirus and cucumbovirus groups are the P1-HCPro component of tobacco etch virus (TEV) (US Patent 5,939,541) and the 2b component of cucumber mosaic virus (CMV).

The entire nucleotide sequence of CMV RNA2 and TEV are available from GenBank under accessions #U20218 and #M15239. Based on these sequences, the open reading frames corresponding to 2b and P1-HCPro components are isolated using standard methods from cDNA synthesized *in vitro* using TEV or CMV-infected plant material or purified viral particles. These sequences are then cloned in the correct orientation into the intermediate cloning vector pER5271 or other similar vector that functionally links the viral coding regions to the figwort mosaic virus (FMV) promoter, petunia *hsp70* non-translated leader sequence, and *Agrobacterium tumefaciens* nopaline synthase (*NOS*-3') polyadenylation signal. The DNA fragment containing the engineered sequences is then isolated by *BamHI* and *BglII* digestion followed by gel purification and ligated into the *BamHI* site of pBINunc-17 from Example 1 or a similar vector containing *nuo-1*. This results in a binary vector that when introduced into plant cells both

suppresses post-transcriptional gene silencing in the plant and also expresses large quantities of dsRNA corresponding to the *M. incognita* orthologs of *nuo-1* or *unc-1*. Plants or hairy root cultures are transformed with these vectors by standard methods and screening occurs as in Example 1 for enhanced resistance to *M. incognita.*

Example 4

This example describes the use of a viral amplicon containing *M. incognita* sequences to induce silencing of the corresponding *M. incognita* gene upon feeding.

Tomato, which is susceptible to both potato virus X (PVX) and *M. incognita* is used for the creation of transformants containing a viral amplicon having an RNA dependent RNA polymerase such as that described in Angell & Baulcombe, *EMBO J.* 16:3675-3684 (1997). This is accomplished by using the plant transformation vector PVX/GUS described in the above reference which is modified such that it contains *unc-17* gene fragments as described in Example 1 instead of the *GUS* gene. Tomato transformants or hairy root cultures established by *Agrobacterium*-mediated transformation with these vectors (*see, e.g.,* McCormick *et al., Plant Cell Reports* 5:81-84 (1986) and Urwin *et al., Plant J.* 8:121-131(1995)). Plants or cultures are then assayed as in Examples 1 and 2 above for evidence of gene silencing and increased resistance to *M. incognita.*

Example 5

This example describes the isolation of target genes.

A partial cDNA corresponding to the *sec-1* gene previously described by Ray *et al., Mol. Biochem. Parasitol.* 68:93-101 (1994) was isolated by the following method. Total RNA from J2 juveniles of *Meloidogyne incognita* was first prepared using TRIZOL reagent (Gibco/BRL, Rockville MD) according to manufacturer's instructions. Poly A+ RNA was then isolated from 300 μg of total RNA using Dynabeads (Dynal biotech, Oslo Norway) again according to manufacturer's instructions. PolyA+ RNA obtained by this method was reverse transcribed and adapted using the Marathon cDNA amplification kit (Clonetech, Palo Alto CA). To specifically amplify the *sec-1* gene, polynucleotide primers: 5'-gagtgaacaggccgaaaaacttgtc-3' and 5'-atttggaagattggaggagcttga-3' that are complementary to published *sec-1* sequences were used to amplify a 1000 base pair sequence using the diluted *M. incognita* cDNA as a

template for amplification. This 1000 base pair sequence corresponded to nucleotides 378-1378 of the *sec-1* coding sequence. The amplification products were cloned into the pCRII-TOPO cloning vector (Invitrogen, Carlsbad CA) and then sequenced. Clones in either orientation were obtained in many cases to allow *in vitro* transcription of either sense or antisense transcripts from the T7 RNA polymerase promoter sequence present in the pCRII-TOPO vector.

Other cDNAs from *M. incognita* were isolated the same way except for a 358 bp sequence with similarity to ubiquinone oxidoreductase designated *Minuo-1*. *Minuo-1* was isolated based on similarity to other ubiquinone oxidoreductase genes using the methods described in Example 2. Degenerate polynucleotide primers corresponding to conserved regions of *nuo-1* were synthesized corresponding to the following sequences: 5'-ctgatgaaggagaaccaggaacntgyaarga-3' and 5'-aaacagtcctatatcagctggraanggngg-3'. Specific *nuo-1* sequences were amplified using these degenerate primers from *M. incognita* genomic DNA isolated from J2 juveniles. The PCR products were then cloned into the pCRII-TOPO vector and sequenced. Sequence analysis yielded the following sequence information.

Minuo1-6

CTTTGAAGGAGAACCAGGAACATGTAAGGACCGCGAGATCATG
CGCCACGACCCTCACAAGCTCATCGAGGGCTGCCTGATCGCCGGCCGTGCCA
TGGGAGCTCGAGCCGCCTATATCTACATCCGCGGCGAGTTCTACAACGAGTCT
TCAAACATGCAACTGGCCATTAACGAGGCCTACAAGGCCGGTCTCATCGGAA
AGAACGCGTGCGGCTCCGGATACGACTTTGATGTCTATATGCATAGGGGTGCT
GGCGCGTACATTTGTGGCGAGGAGACGGCGCTTATTGAGTCGCTCGAAGGCA
AGCAGGGTAAACCGCGTCTTAAGCCTCCCTTCCCAGCTGATATAGGACTGTTT

Genes from *Manduca sexta* were isolated in the same way as *sec-1* except that polyA+ RNA used for cDNA synthesis was isolated from 3rd instar larvae of *M. sexta*. Table 3 lists the gene sequences isolated from *M. incongnita* and *M. sexta* using these methods.

Example 6

In this example, a plant-viral inhibitor of gene silencing was used for the purpose of allowing accumulation of sufficient quantities of dsRNA in plant cells to surpass the threshold necessary for triggering of RNA silencing in plant-parasitic nematodes.

In this experiment, a viral suppressor of gene silencing corresponding to the *AC2* gene from African cassava mosaic virus was expressed in plant cells. The *AC2* gene has been described previously as a suppressor of gene silencing by Fondong *et al., J. Gen. Virol.* 81: 287-297 (2000) and Voinnet *et al., Proc Nat'l Acad. Sci U S A.* 96:14147-52 (1999). Using mutagenic primers, *NcoI* and *XbaI* restriction sites were introduced into the 5' and 3' ends respectively of the *AC2* coding sequence. The *AC2* gene was then cloned between the FMV promoter and *nos* polyadenylation signals using unique *NcoI* and *XbaI* sites in the vector.

Another gene cassette containing a test DNA that contained the *LFY* gene of *Arabidopsis thaliana* was made (Weigel *et al., Cell* 69:1163-1166 (1992)). Insertion of the test DNA sequences between two plant promoters flanked by polyadenylation signals created a situation where the test DNA was transcribed bidirectionally to generate both sense and antisense messages. The *AC2* expression cassette and the test DNA expression cassette were then cloned together using unique *BamHI* and *BglII* sites into an intermediate vector and then between left and right T-DNA borders of the plant transformation vector pWTT2144 using a unique *BamHI* site to generate the vector pCT645. The region that is between the left and right T-DNA border of this vector is shown in Figure 2. Additional negative control vectors were created in a similar manner either lacking a second gene entirely (pCT616) or containing a sequence similar in size to *AC2* that was not predicted to suppress gene silencing (pCT660).

These vectors were transformed into *Agrobacterium rhizogenes* isolate 15843 from the American Type Culture Collection and tomato cotyledon explants were transformed using the methods described in Urwin et *al., Plant J.* 8:121-131(1995).

After subculturing hairy roots for four weeks in liquid media in the presence of kanamycin to select for transformed tissue, RNA was isolated from several independently transformed root lines. RNA was extracted by grinding in liquid nitrogen followed by extraction with SDS buffer and phenol/chloroform. RNA was precipitated with lithium acetate and screened for the presence of *LFY* sequences by RNA blot analysis of both low and high molecular weight RNA species with a [^{32}P]-labeled polynucleotide probe. Comparison of high molecular weight RNA species hybridizing to the probe indicated that the hairy root lines transformed with pCT645 accumulated significantly more intact *LFY* RNA than the lines transformed with the two control constructs (Figure 3). Significantly more message degradation in the control samples (presumably the result of PTGS) is seen in panels A and B relative to C. This data is

consistent with RNA blot analysis of low molecular weight RNAs derived from the same transgenic hairy root lines using the method of Hamilton & Baulcombe, *Science* 286:950-952. Analysis of this low molecular weight RNA, shown in Figure 4, detects the presence of approximately 23 base RNAs that positively hybridize with riboprobes derived from the *LFY* test sequence. These small 21-23 bp RNAs are indicative of PTGS and are present to a greater degree in the control lines that lack the *AC2* gene.

Example 7

To examine the timing and degree of susceptibility to dsRNA of a plant parasitic nematode, *Meloidogyne incognita* was treated with dsRNA corresponding to the *sec-1* gene. Double-stranded RNA was synthesized from plasmid templates in the pCRII-TOPO vector that had been digested with *BamHI* to linearize the plasmid after the *sec-1* sequence. RNA was transcribed and annealed *in vitro* using the Megascript T7 RNA transcription kit (Ambion, Austin TX). Analysis of RNA mobility by agarose gel elecrophoresis of RNA aliquots corresponding to sense, antisense, and annealed sense plus antisense strands demonstrated that the majority of the RNA was double stranded. The dsRNA was quantitated by UV absorption and incubated in the presence of 1500 second stage juvenile worms of *M. incognita* at a final concentration of between 1 and 4 μg/μl in a total volume of 15 microliters of water on parafilm in a humid chamber. After an overnight incubation of between 16 and 18 hours, the worms were collected and frozen. RNA was then isolated from the frozen samples using TRIZOL reagent. For each of the treatments *sec-1* message was quantitated using an ABI Prism 7700 sequence detector with Taqman SYBR green and reverse transcription kits (Applied Biosystems, Foster City CA). Briefly, worm RNA was first reverse transcribed using random hexamers to prime cDNA synthesis, and then *sec-1* and 18S ribosomal sequences were amplified in separate tubes using the following primers pairs: Misec-1 274F, 5'-gaggaagcaaccaaagaacttaattta-3'; Misec-1 359R, 5'-gcattttcaatacgcaaattttctaac-3'; 18S ribo-13F, 5'-caatttaatcgcagtggcttga-3'; 18S ribo-85R, 5'- gatccagcagcaggttcacc-3'. The primers used for amplification of *sec-1* were designed from a region of the gene outside of the sequence used to produce dsRNA to avoid the possibility of obtaining artifactual expression data due to amplification of introduced RNA. The Misec-1 274F primer also encompassed an intron/exon boundary to avoid the possibility of accidental amplification of contaminating genomic DNA sequences. Samples were subjected to real time

quantitative PCR using recommended conditions for relative quantitation of gene expression.

The results of this experiment are presented in Table 4. Incubation of worms in the presence of dsRNA led to significant reductions of message levels at all concentrations tested up to a maximal level of 96%. Expression levels between treatments were normalized relative to 18S ribosomal RNA levels amplified from the same cDNA. Water treated control worms were given an arbitrary expression level of 1.0.

Example 8

In this experiment, susceptibility of *Manduca sexta* to RNAi was determined by treating larvae with dsRNA by either feeding or by direct injection.

To feed worms dsRNA, an insect artificial diet commercially available from ICN (Aurora, Ohio) is used. Double stranded RNA dissolved in water is then added to small portions of food of known mass, and newly hatched *Manduca* larvae are placed on the food. After a designated time, RNA is isolated and the amount of endogenous message corresponding to the dsRNA used for treatment is quantitated by RNA blot analysis or by using RT-PCR with sequence specific primers as is described in Example 7.

For injection of dsRNA, larger 4th instar larvae are used and injected between 4th and 5th prolegs with 10 μl of dsRNA solution from a 30 gauge needle. Twenty four hours later RNA from injected animals is isolated and endogenous message is quantitated as above.

Results from these experiments show that with dsRNA sequences derived from three separate genes, injection into *M. sexta* larvae leads to substantial decreases in expression of the endogenous gene relative to mock inoculated controls (Figure 5). There is also substantial hybridization to low molecular weight RNA that may be indicative of PTGS.

Table 3. Gene fragments isolated from *Meloidogyne incognita* and *Manduca sexta*

				pCRII-TOPO clones	
Organism	*Gene product*	*Genbank*	*Fragment size*	*T7 -strand*	*T7+strand*
M. SEXTA	Trypsin	L16806	791		pCT670-7
	Chymotrypsinogen	L34168	922	pCT671-7	pCT671-2
	V-ATPase	AJ249390	1294	pCT682-5	pCT693-5
	S6 ribosomal protein	U64795	769	pCT694-3	pCT683-5
M. INCOGNITA	SEC-1	U09180	1000	pCT669-9	pCT669-2
	Collagen	U68729	969		pCT684-5
	Acetylcholinesterase	AF075718	1500		pCT676-2
	β-1,4-endoglucanase	AF100549	1300	pCT677-1	pCT677-10
	Superoxide dismutase 5'	AW571222	220	pCT673-3	
	Superoxide dismutase 3'	AW571222	400-600	pCT674-9	pCT691-2
	Ubiquinone oxidoreductase	none	376		pCT598

Table 4. Amount of *sec-1* message in *M. incognita* second stage juveniles treated with dsRI

treatment	*sec-1* µl H_20 control[a]	18S µl H_20 control	*sec-1* normalized to 18S
H_20	0.97	1.06	
	0.91	0.87	
	0.87	1.02	
	1.02	1.26	
	1.06	1.05	
	0.98	0.74	
	0.81	0.90	
	1.26	1.22	
average	0.99±0.14[b]	1.01±0.18	0.97±0.22
1 µg/ul dsRNA	0.08	3.32	
	0.18	3.23	
	0.15	4.20	
	0.18	3.89	
	0.20	3.23	
	0.24	4.62	
	0.15	4.33	
	0.21	4.53	
average	0.17±0.05	3.92±0.59	0.044±0.014

2 µg/ul dsRNA	0.10	0.56	
	0.11	0.65	
	0.07	0.85	
	0.18	0.60	
	0.14	0.65	
	0.13	0.77	
	0.05	0.61	
	0.25	0.53	
average	0.13±0.06	0.65±0.11	0.20±0.10
4 µg/ul dsRNA	0.12	0.28	
	0.11	0.37	
	0.08	0.30	
	0.15	0.32	
	0.15	0.30	
	0.14	0.37	
	0.29	0.34	
average	0.15±0.07	0.33±0.04	0.46±0.21

[a]quantitated based on comparison with a standard curve generated from dilution of cDNA produced from water treated control worms.

[b]averages values are reported ± standard deviation.

WHAT IS CLAIMED IS:

1. A method of conferring plant pest resistance to a plant cell, the method comprising the step of expressing in the plant cell a first nucleic acid comprising a first expression cassette comprising a first plant promoter operably linked to a first plant pest nucleic acid that produces a first transcript, which forms a double stranded RNA, either with itself or with a second complementary transcript, wherein the first nucleic acid further comprises means for enhancing RNA accumulation, thereby conferring plant pest resistance to the plant cell.

2. The method of claim 1, wherein the first nucleic acid is DNA.

3. The method of claim 1, wherein the first nucleic acid is RNA.

4. The method of claim 1, wherein the plant pest is an insect pest.

5. The method of claim 4, wherein the insect pest is a piercing-sucking pest.

6. The method of claim 5, wherein the insect pest is a sap-sucking pest or a chewing pest.

7. The method of claim 6, wherein the chewing insect pest is *Manduca sexta.*

8. The method of claim 1, wherein the plant pest is a parasitic nematode.

9. The method of claim 1, wherein the first transcript is self-complementary.

10. The method of claim 1, wherein the first nucleic acid further comprises a second expression cassette.

11. The method of claim 10, wherein the second expression cassette is the means for enhancing RNA accumulation, comprising a second plant promoter operably linked to a viral nucleic acid encoding a protein that enhances accumulation of double-stranded RNA in the plant cell.

12. The method of claim 11, wherein the viral nucleic acid encodes HCPro or AC2 protein.

13. The method of claim 10, wherein the second expression cassette is the means for enhancing RNA accumulation, comprising a second plant promoter operably linked to a viral nucleic acid encoding an RNA dependent RNA polymerase, wherein the first transcript is operably linked to an RNA dependent RNA polymerase promoter.

14. The method of claim 13, wherein the RNA dependent RNA polymerase is from potato virus X or tobacco mosaic virus.

15. The method of claim 10, wherein the second expression cassette comprises a second plant promoter operably linked to a second plant pest nucleic acid that produces a second transcript complementary to the first transcript.

16. The method of claim 1, wherein the first plant pest nucleic acid comprises a gene, or a fragment of said gene, required for nervous system function.

17. The method of claim 1, wherein the first plant pest nucleic acid comprises a gene, or a fragment of said gene, required for embryonic survival.

18. The method of claim 1, wherein the first plant pest nucleic acid encodes a protein selected from the group consisting of a vesicular acetylcholine transporter protein, a choline acetyltransferase, or a ubiquinone oxidoreductase.

19. The method of claim 1, wherein the plant pest is a parasitic nematode selected from the group consisting of a cyst nematode or a root-knot nematode.

20. The method of claim 19, wherein the parasitic nematode is *Heterodera glycines*, *Globodera pallida*, *Globodera rostochiensis*, or *Meloidogyne incognita*.

21. The method of claim 1, further comprising expressing in the plant cell a second nucleic acid comprising a second expression cassette.

22. The method of claim 21, wherein the second expression cassette comprises a second plant promoter operably linked to a second plant pest nucleic acid that produces a second transcript complementary to the first transcript.

23. The method of claim 22, wherein the first or the second nucleic acid is introduced into the plant cell through sexual reproduction.

24. The method of claim 1, wherein the plant promoter is a tissue specific promoter.

25. The method of claim 24, wherein the plant promoter is active in plant parasitic nematode feeding cells.

26. The method of claim 1, wherein the plant promoter is a CaMV 35S promoter.

27. The method of claim 1, wherein the double stranded RNA is about 25 to 100 bp in size.

28. The method of claim 1, wherein the plant is selected from the group consisting of pepper, tomato, squash, banana, strawberry, carrot, bean, cabbage, beet, cotton, grape, pea, pineapple, potato, soybean, yam, and alfalfa.

29. The method of claim 1, wherein the first transcript comprises a premature stop codon that inhibits translation of the first transcript.

30. The method of claim 1, wherein the double-stranded RNA has a duplex region that is at least about 50 bases in length.

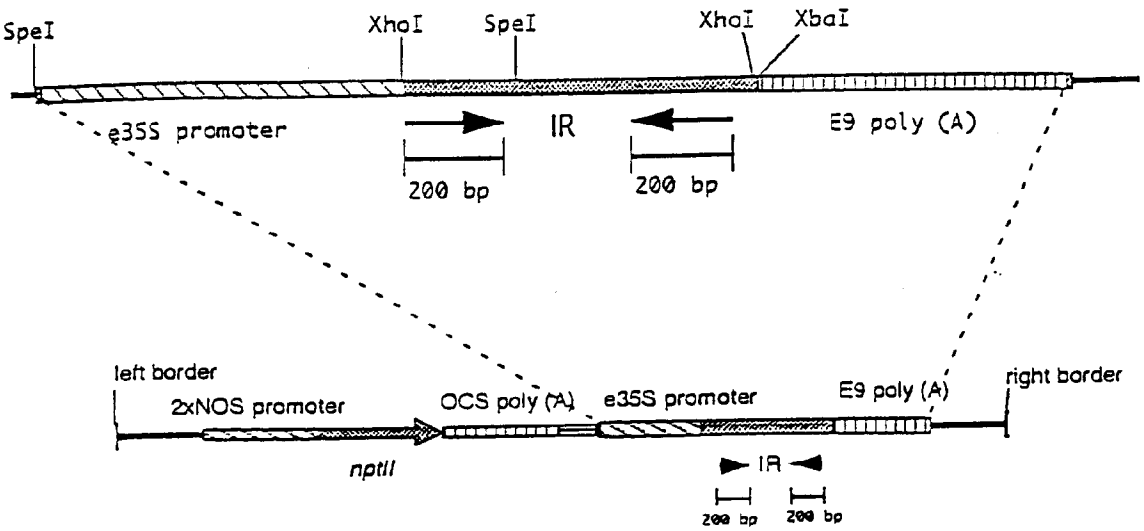
31. The method of claim 1, wherein the first expression cassette is a plant viral vector.

32. A method of reducing expression of a plant pest mRNA, the method comprising the step of contacting the plant pest with a double stranded RNA that is either self complementary or hybridizes with a second complementary transcript, thereby reducing expression of the plant pest mRNA.

33. The method of claim 32, wherein the plant pest is selected from the group consisting of insect pests and parasitic nematode pests.

34. The method of claim 33, wherein the insect pest is a piercing-sucking pest.

35. The method of claim 34, wherein the insect pest is a sap-sucking pest or a chewing pest.


SpeI
XhoI
SpeI
XhoI
XbaI
e35S promoter
IR
E9 poly (A)
200 bp
200 bp
left border
2xNOS promoter
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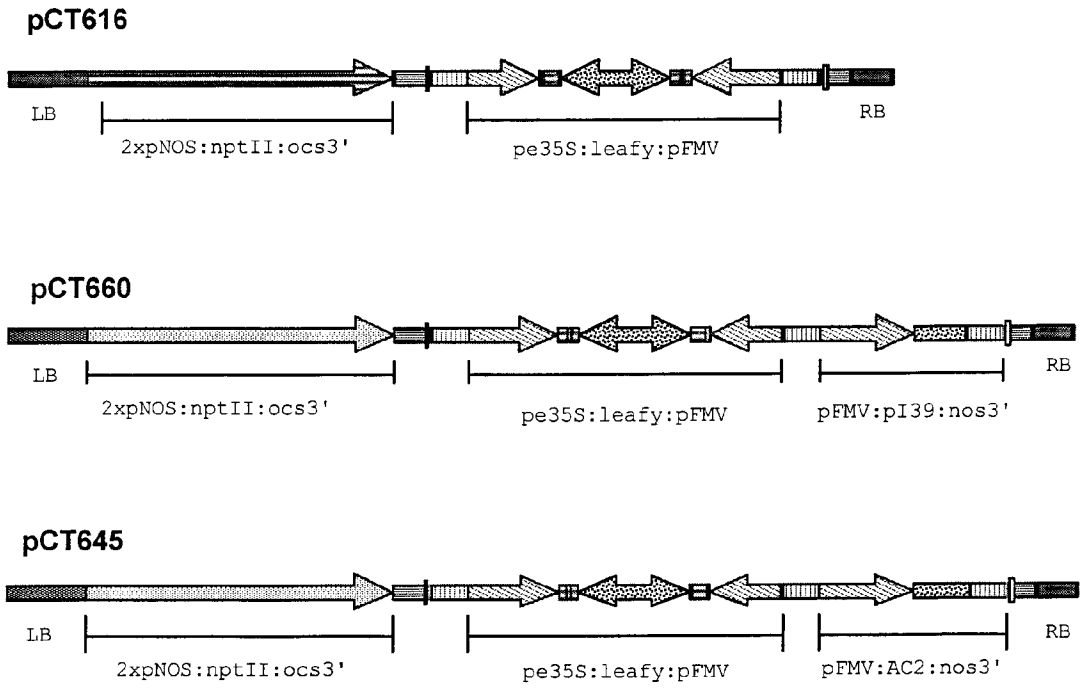
e35S promoter
E9 poly (A)
right border
nptII
IR
200 bp
200 bp


pCT616
LB
RB
2xpNOS:nptII:ocs3'
pe35S:leafy:pFMV
pCT660
LB
RB
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pe35S:leafy:pFMV
pFMV:pI39:nos3'
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pe35S:leafy:pFMV
pFMV:AC2:nos3'

Figure 2

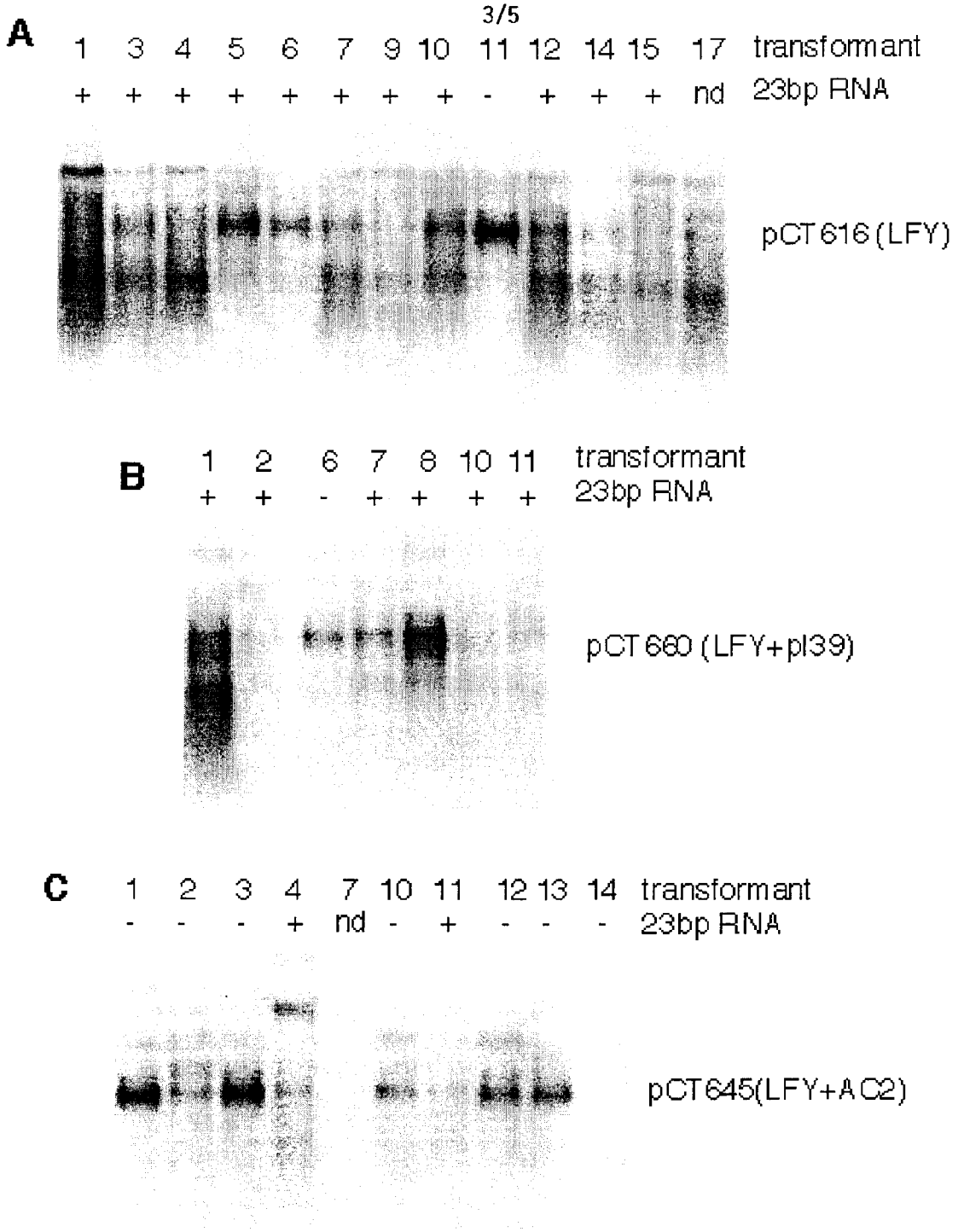
A
1 3 4 5 6 7 9 10 11 12 14 15 17 transformant
+ + + + + + + + - + + + nd 23bp RNA
pCT616 (LFY)
B
1 2 6 7 8 10 11 transformant
+ + - + + + + 23bp RNA
pCT680 (LFY+pl39)
C
1 2 3 4 7 10 11 12 13 14 transformant
- - - + nd - + - - - 23bp RNA
pCT645(LFY+AC2)

Figure 3

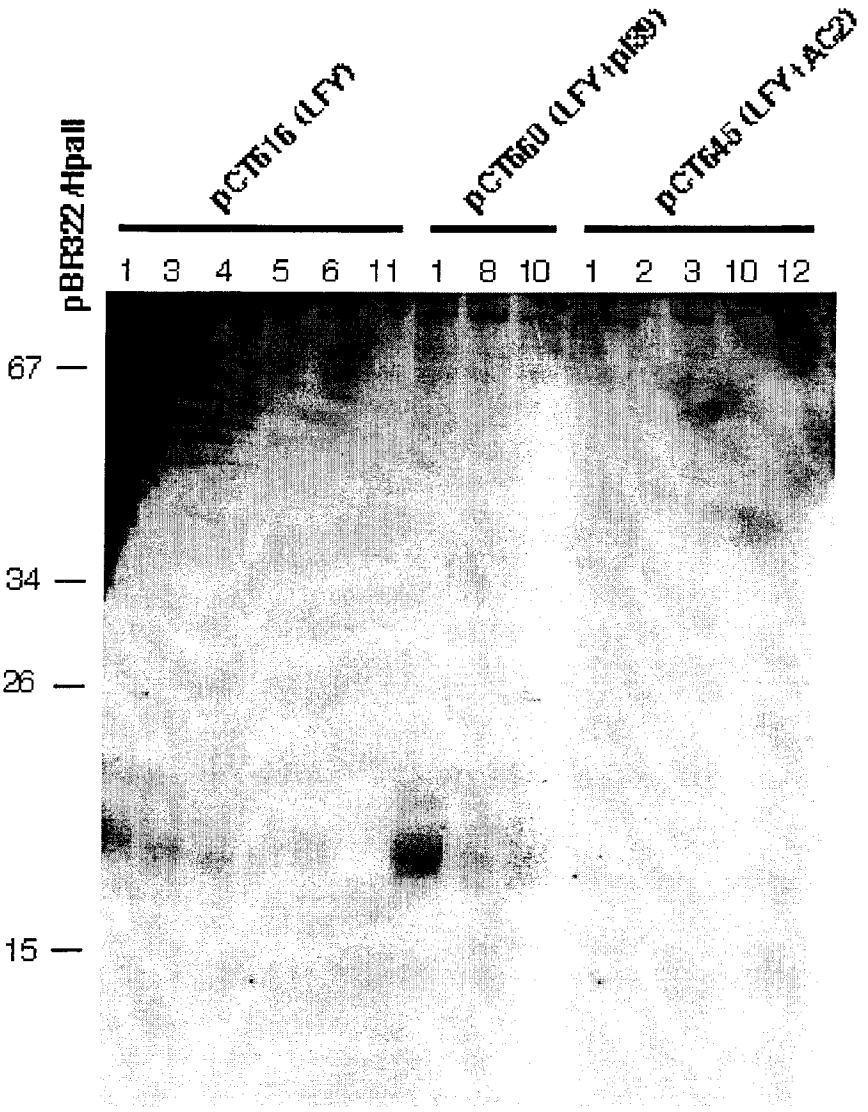
pBR322 /HpaII
pCT616 (LY)
pCT660 (LY+pES9)
pCT645 (LY+AC2)
1 3 4 5 6 11 1 8 10 1 2 3 10 12
67
34
26
15

Figure 4

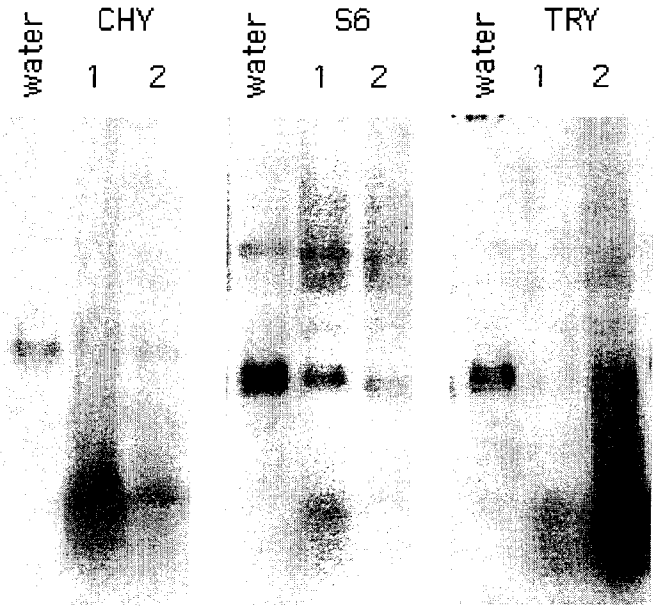

Figure 5